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14. ABSTRACT
Breast cancer develops from breast mucosa and breast mucosa has intact immune system to maintain epithelial integrity. In this study our goal was to study the immune subsets associated with breast mucosa and develop the strategies to populate mucosa with immune effectors in order to prevent breast cancer. Data obtained from our studies suggest that T cells constitute the majority of immune cells in breast mucosa and this includes conventional CD4 T cells, CD8+ T cells and significant fraction of unconventional double positive (DP) CD4+CD8+ T cells. We also observed that intramammary immunization induces antigen-specific immune responses in breast mucosa. Currently, studies are being done to characterize these double positive T cells to determine whether these are regulatory or cytotoxic in nature and their role in prevention of breast cancer. In addition to this, we are also investigating the ability of intramammary immunization in prevention of breast cancer and the feasibility of translating this approach into preventive breast cancer vaccine setting.

15. SUBJECT TERMS- Intraepithelial lymphocytes, Theilers murine encephalomyelitis virus, rat neu, intra mammary immunization, T cells

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Introduction: The prevention of breast cancer is an active area of research in many countries and several different agents have been examined, notably estrogen antagonists. While these agents can be effective in high risk individuals, they only reduce the risk of specific subtypes of cancer and moreover these currently available drugs are associated with significant side effects and must be consumed daily and over years. Thus, an alternative approach to primary prevention of breast cancer is to train the mammary gland mucosal immune system to recognize and destroy potentially malignant cells based on overexpression of known tumor antigens. Rationale for this approach comes from the fact that breast cancer originates from a mucosal tissue which is endowed with large number of immune effectors and prior reports based on the effect of commensal bacteria on gut mucosa which suggests that it's feasible to educate gut mucosa in generating effective mucosal immunity. Our overall goal is to develop a preventative vaccination strategy to reduce the incidence and mortality from breast cancer based on training the mucosal immune system to detect potentially malignant cells early in the course of the disease. One important first step towards attaining this unique objective is to have a comprehensive knowledge about the breast mucosal immune system i.e. a profile of immune effectors, and their biology in the breast mucosa, including both normal and hyperplastic breast mucosal tissue. Our goal in the present study is to understand the immune effector biology of breast mucosa (normal and cancer prone) and identify strategies which can enhance the infiltration of antigen-specific immune effectors targeting tumor cells in breast mucosa. As a part of this plan, we intend to use the transgenic mouse model of human breast cancer. We hypothesize that the mucosal immune system of the mammary gland can be modified to detect and eliminate potentially malignant cancer precursor cells. A thorough understanding of the immunity in breast mucosa will enable the design of appropriate vaccination strategies aimed at generating persistent mammary gland homing anti-cancer antigen-specific immune effectors. To test this general hypothesis we propose the following specific aims: Specific Aim 1: To determine the immune cell subsets in normal and hyperplastic breast in murine breast cancer models. Specific Aim 2: To determine whether immunization of mice with tumor antigen modifies the immune cell subsets and leads to persistent trafficking of tumor antigen-specific T and B cells into the mammary gland. Specific Aim 3: Determine an optimal oral vaccine approach able to minimize hyperplasia.

Progress report

Task 1: To determine immune cell subsets in normal breast mucosa compared to the small intestine. The mucosal immune system in the gut consists of unique populations of T cells, including self-antigen specific CD8α T cells, in addition to conventional effector T and B cells. One of our goals in this study was to determine if the mucosal effector immunity is similar in immune content to normal gut mucosa, including T and B cell phenotypes and oligoclonality of T cells.

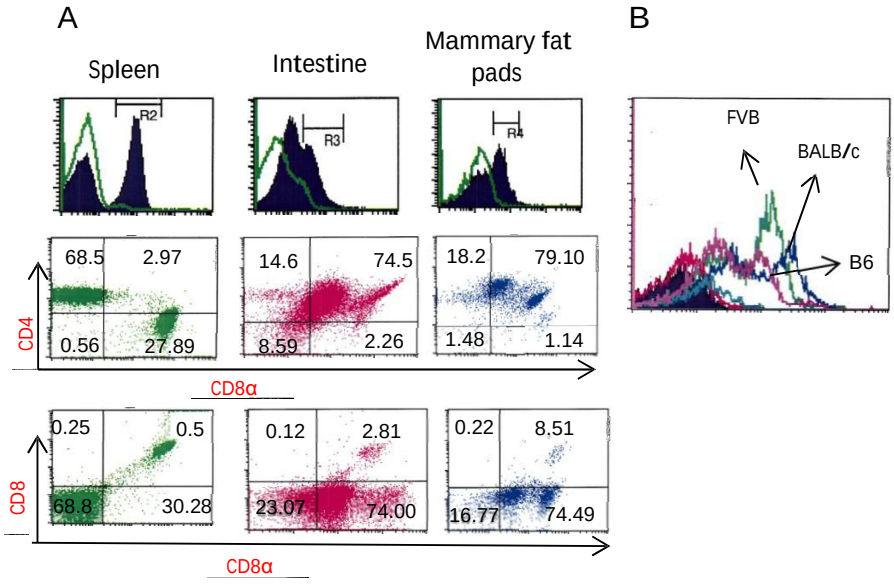

Figure 1.T cell population in the intestine and mammary fat pads of BALB/c mice. (A) Shown are the histograms of cells gated on TCRαβ (upper panel). Shown are dot plots representative of double positive T cells (CD4+CD8α+) (middle panel) and CD8αβ (lower panel) T cells in the spleen, intestine and mammary fat pads. (B) Histogram showing the expression of CD8α on the mammary fat pad derived double positive T cells obtained from different strains of mice.

In this first set of experiments we wanted to determine whether the immune cell subsets obtained from breast mucosa (mammary fat pads) are similar in phenotype and function when compared to the immune subsets obtained from gut mucosa (intestine) and spleen. For this purpose tissues were obtained from three different strains of mice i.e. C57BL/6, BALB/c and FVB. Single cell suspensions were obtained from mammary pads, small bowel and spleen. These single cell

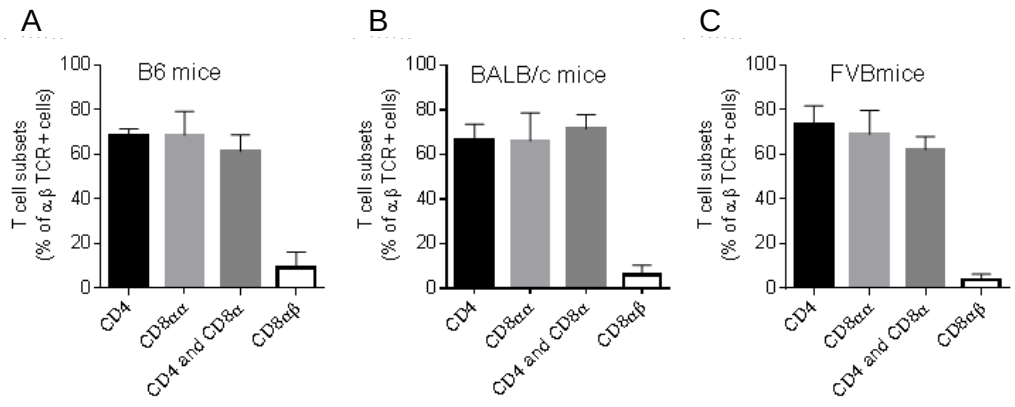

Figure 2.TCRαβ+ T cell subsets in C57BL/6, BALB/c and FVB mice. Single cell suspensions obtained from mammary fat pads were stained with anti-TCRαβ-FITC, anti-CD4-APCcy7, anti-CD8α-PerCPCy5.5, and anti-CD8β-PE. The CD4, CD8 expression phenotype in each animal was determined after gating on TCRαβ+ cells. The results are shown as mean (± s.e.m) percentage of T cell subsets and data shown is the representative of one of three experiments with similar results.

suspensions were stained with antibodies specific for T lymphocyte subsets, B lymphocytes and dendritic cells (DCs). It was observed that majority of immune cell subset population in the breast mucosa is T cells. Further analysis of T cell subsets revealed the existence of unconventional T cells CD4+CD8+ αβ T cells in both intestine and mammary fat pads (Fig. 1). It was observed that majority of the T cell subsets that exist in the mammary gland are indeed these unconventional double positive T cells and the percentage of these double positive (DP) T cells across different species were similar suggesting that the haplotype of the different strains of mice didn't have any effect in altering this cell population in mammary fat pads (Fig. 2). In the next step, we analyzed the expression of integrins, $\alpha_4\beta_7$ and $\alpha_E\beta_7$, on these double positive T cells. It was observed that 3% (± 2) of DP cells obtained from intestine and 7% (± 2.06) of DP cells obtained from mammary fat pads express $\alpha_4\beta_7$. Also, we observed that 10% (± 2) of DP cells obtained from intestine and 19% (±1) of DP cells obtained from mammary fat pads express $\alpha_E\beta_7$ (Fig. 3). Studies by Huang Y et al (1) and Olivares-Villagomez D et al (2), showed that thymus leukemia antigen in the gut mucosa selects for the presence of CD8 T cells that have memory phenotype i.e. CD8αα+CD8αβ (1-3). As our results showed that majority of T cells in the breast mucosa (mammary fat pad derived cells) are double positive T cells that express high levels of CD8α, in the next step we wanted to determine whether breast epithelial cells express thymus leukemia antigen unlike the epithelial cells in the intestine. For this single cell suspensions obtained from mammary fat pads and intestine were stained with anti-EpCAM antibody (marker for epithelial cells) and anti-TL-antibody. As shown in Fig. 4, it was observed that EpCAM positive cells obtained from intestine and mammary fat pads expressed high levels of TL antigen compared to cells that were

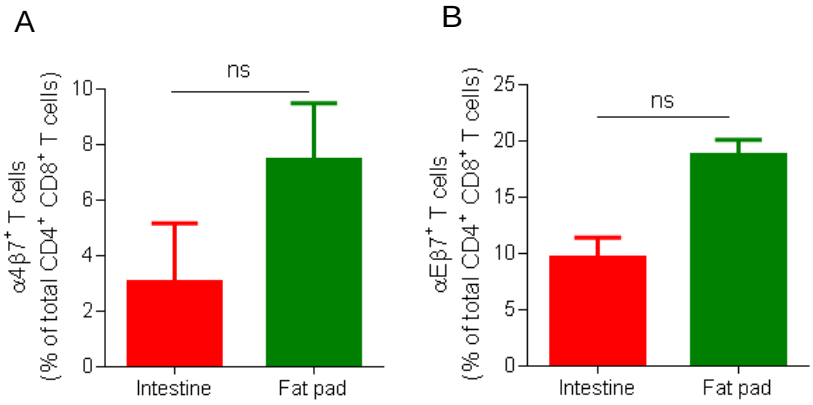

Figure 3. Expression of integrins on mucosa associated double positive T cells. Shown are mean (± s.e.m) numbers of double positive (CD4+CD8α+) T cells which are $\alpha_4\beta_{7+}$ (A) and $\alpha_E\beta_{7+}$(B) in the intestine and mammary fat pads of BALB/c mice. Data is Shown are the means of the (± s.e.m) triplicate samples pooled from 3 mice. Data is shown as mean (±s.e.m) of three repeated experiments with similar results.

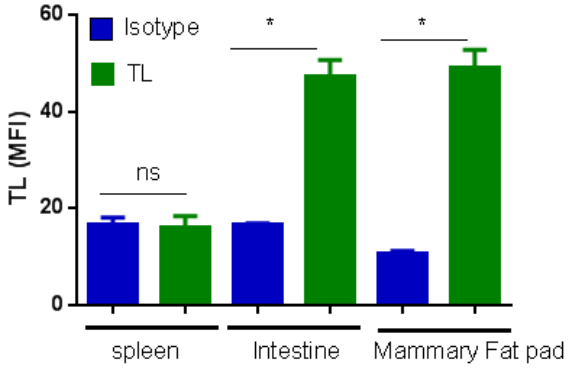

Figure 4. Breast epithelial cells express thymus leukemia (TL) antigen in a fashion similar to intestine epithelial cells. Intraepithelial lymphocytes (IELs) obtained from intestine and single cell suspensions obtained from mammary fat pads were stained with anti-EpCAM-PE antibody and anti-TL-FITC antibody. Shown is the mean fluorescence intensity (MFI) of TL expression on EpCAM+ cells. Data is shown as mean (±s.e.m) of three replicates.

obtained from spleen. These findings suggest the presence of T cells with memory phenotype i.e. CD8αα+CD8αβ+ that were reported in gut mucosa are also present in breast mucosa. Studies by Reimann J et al (4) and Desfrancois J et al (5) showed that presence of DP cells and

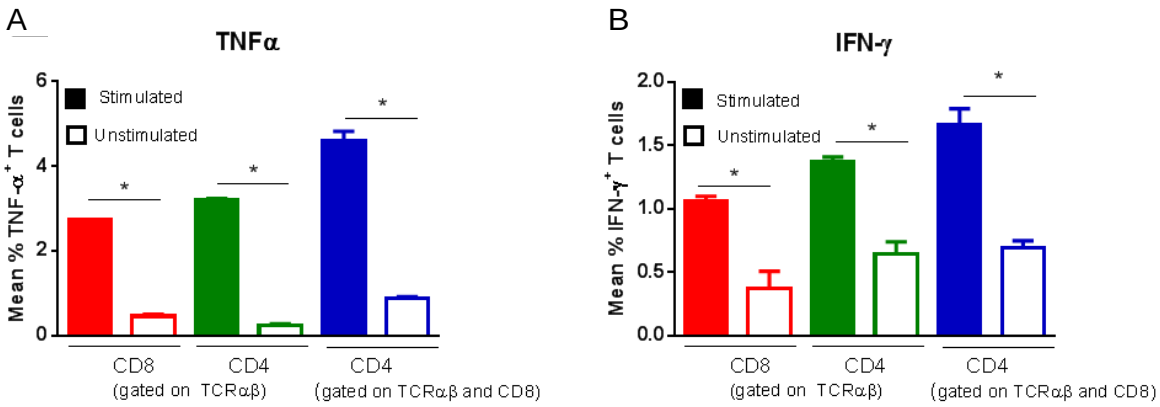

Figure 5. TNF-α and IFN-γ secretion by T cells derived from mammary fat pads. Single cell suspensions obtained from mammary fat pads were stimulated in vitro with concavalin A for 6 hrs. in the presence of Golgiplug. Cells were fixed using BD cytofix/cytoperm fixation/permeabilization kit and analyzed for lymphocyte markers and intracellular TNF-α, IFN-γ. Data is shown as mean (±s.e.m) of three replicates.

their function in cancer patients is crucial especially in the context of cancer immunotherapies. It was also observed in these studies that the cytokine profile associated with these DP cells indicates that their function is similar to cytotoxic T cells. Our results show that (Fig. 5) these DP cells obtained from mammary fat pads express high levels of TNF-α and IFN-γ suggesting that their function is similar to the function of DP cells that were reported in prior studies. Currently, studies are being done to further characterize these breast mucosa associated DP cells and their role in protecting transgenic mouse models of human breast cancer from spontaneous neoplasms.

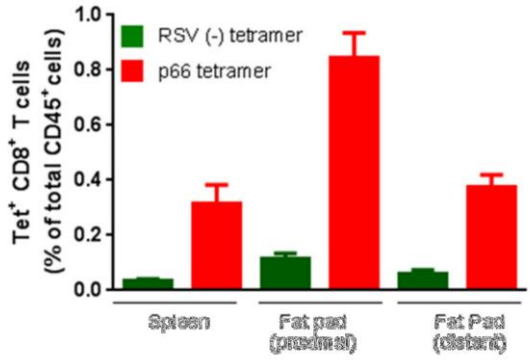

Figure 7. Intramammary immunization induces local antigen specific tetramer responses. BALB/c mice were immunized with a multi-peptide vaccine C3L3N1 via intramammary route and single cell suspensions obtained from mammary fat pads , spleens were stained with anti-CD45-FITC ab, anti-CD8-APC ab and PE-labeled N1 tetramer/RSV tetramer (-ve control). Shown are the percent tetramer positive CD8 T cells out of total CD45+ cells in mammary fat pads. Data is shown as mean (± s.e.m) tetramer (N1/RSV) positive CD8 T cells and is representative of three independent experiments.

Task 2: To determine if local immune induction generates tumor antigen-specific effector lymphocytes. In this specific task, by incorporating findings from task 1 which showed the presence of T cells with memory phenotype in breast mucosa, we wanted to address the fundamental hypothesis as to whether the mammary gland mucosal immune microenvironment can be modified with vaccine to enrich for tumor-antigen-specific lymphocytes and/or T cells with memory phenotype. Given the fact that inducing locally leads to imprinting that facilitates

homing of activated lymphocytes into mucosa associated epithelium, in this task our goal was to induce local immunity by intramammary (Ima)

immunization with tumor antigens delivered in the form of peptide vaccine and attenuated virus vaccine (Theilers virus). For this, initially we tested whether local delivery (intramammary immunization) of a multi-peptide vaccine (a vaccine which was observed to be immunogenic and cause regressions in transplanted breast cancer model) targeting antigens of tumor infrastructure can induce immune response in breast tissue. Normal BALB/c mice were immunized with three doses of multi-peptide (peptides C3, L3 and N1) vaccine on day 0 (in CFA) and on day 3, 6 (in IFA). These doses were delivered via intramammary route and subcutaneous (SQ) route (base of tail). Immune responses against the peptides that were used in vaccine were determined by ELIspot assay. As shown in Fig. 6, intramammary immunization with peptide vaccine generated antigen-specific responses in breast mucosa. Single cell suspensions obtained from mammary fat pads of mice immunized with vaccine through Ima and SQ routes showed increase in IFN-γ production. Notably, mammary fat pad cells obtained from mice immunized via Ima route showed increase in response to concavalin A suggesting the presence of more T cells in these fat pads compared to the fat pads in the mice that were immunized subcutaneously. As expected splenic CD8 T cells obtained from mice that were immunized subcutaneously showed significant increase in IFN-γ production whereas similar response was not observed in splenic CD8 T cells obtained from mice that were immunized through Ima route. Ability of Ima route of immunization to induce local immune responses particularly against immunogenic peptide N1 in vaccine was further confirmed by tetramer assay. As shown in

Fig.7, single cell suspensions obtained from mammary fat and spleens of immunized mice showed when stained for CD45, CD8 markers

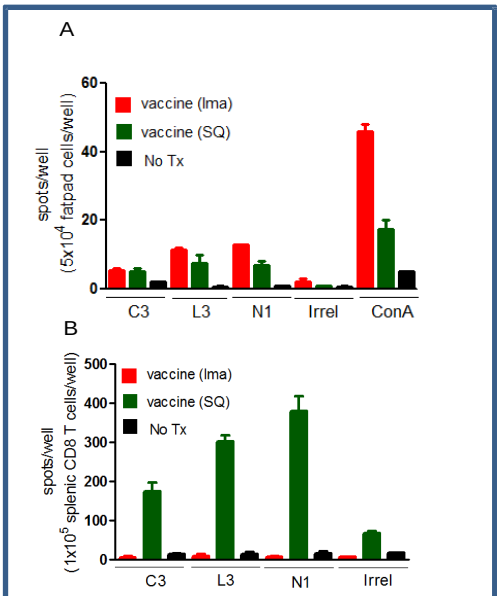

Figure 6. Intramammary immunization induces local antigen-specific immune responses. BALB/c mice were immunized with a multi-peptide vaccine C3L3N1 via intramammary route and sub cutaneous route and single cell suspensions obtained from fat pad cells (A), CD8 T cells obtained from spleen (B) were analyzed for peptide-specific responses using ELIspot. Shown are the results of IFN-γ production by mammary fat pad cells (A) and splenic CD8 T cells (B) in response to C3, L3 and N1 peptides pulsed P815 cells. Data is shown as mean (±s.e.m) of triplicates and is the representative of one of three experiments with similar results

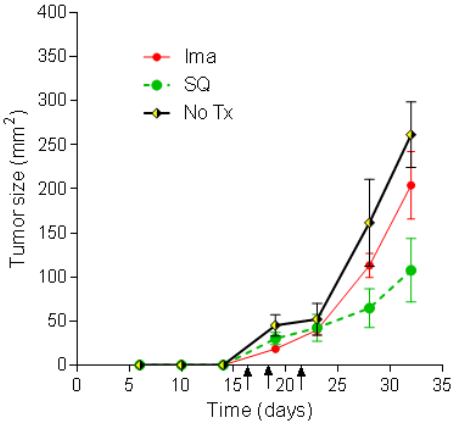

Figure 8. Intramammary immunization did not induce protection against subcutaneously transplanted tumors. BALB/c mice were injected subcutaneously with $1x10^5$ TUBO cells, and on day 16, 19 and 22 post-tumor implantation mice received C3L3N1 peptide vaccine subcutaneously (SQ) and by intramammary route (Ima) as shown by arrows. Tumor growth was measured (two opposing diameters) and recorded every other day. Results are presented in size as mm^2, calculated by multiplying the two diameters for each tumor.

along with p66 (N1) tetramer, an increase in tetramer-specific CD8 T cells was observed in fat pad and spleen derived cells. High number of p66 tetramer-specific CD8 T cells was observed in fat pad cells that were obtained from site that is close to immunization compared to site that was distant from immunization. Also, as shown, no differences in RSV tetramer-specific (negative control) responses were observed in both groups of mice. In our preliminary studies we observed that SQ immunization of tumor (TUBO)-bearing BALB/c mice with multi-peptide vaccine C3L3N1 induces regression of tumors. We wanted to determine whether Ima immunization will have similar effect on tumor (TUBO)-bearing BALB/c mice. As shown in Fig. 8, we observed that Ima immunization did not induce tumor regression in BALB/c mice that were implanted with TUBO tumors on right flank. These results suggest that immune responses elicited by Ima immunization do not have off target effects. Next, we wanted to determine whether Ima immunization induces immune responses and protection against spontaneous mammary neoplasms in neu-transgenic mouse. As shown in Fig. 9A single cell suspensions obtained from mammary fat pads of mice immunized with vaccine through Ima and SQ routes showed increase in IFN-γ production. Also, splenic CD8 T cells obtained from neu-transgenic mice immunized subcutaneously showed significant increase in IFN-γ production whereas similar response was not observed in splenic CD8 T cells obtained from neu-transgenic mice immunized through Ima route (Fig. 9B). Even though Ima immunization in neu-transgenic mice induced immune responses against multi-peptide vaccine,

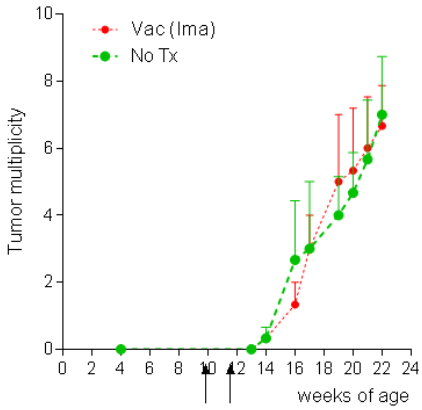

Figure 10. Intramammary immunization did not delay spontaneous neoplasms in neu transgenic mice. BALB/c-neu transgenic mice were immunized with 3 doses of C3L3N1 peptide vaccine by intramammary route (Ima) between weeks 9-11 as shown by arrows. All animals were monitored for tumor appearance by manual examination of the mammary glands every 5d. Measurable masses >2mm diameter were regarded as tumors. Data is shown as mean number of tumors (tumor multiplicity) in each group which is represented by points. These experiments were repeated twice with similar results.

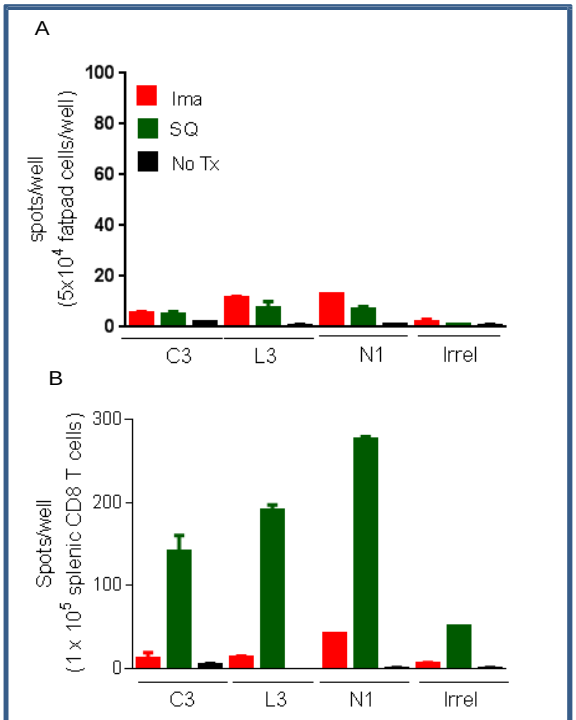

Figure 9. Intramammary immunization induces local antigen-specific immune responses in neu transgenic mice. BALB/c-neu transgenic mice were immunized with a multi-peptide vaccine C3L3N1 via intramammary route and sub cutaneous route and single cell suspensions obtained from fat pad cells (A), CD8 T cells obtained from spleen (B) were analyzed for peptide-specific responses using ELIspot. Shown are the results of IFN-γ production by mammary fat pad cells (A) and splenic CD8 T cells (B) in response to C3, L3 and N1 peptides pulsed P815 cells. Data is shown as mean (±s.e.m) of triplicates and is the representative of one of three experiments with similar results .

it was not able to induce protection against spontaneous mammary neoplasms (Fig. 10). Currently, we are investigating whether addition of booster dose of vaccine and/or change in the schedule of Ima immunization has any effect in inducing protection against spontaneous mammary neoplasms in neu-transgenic mice.

Theilers murine encephalomyelitis virus (TMEV) is naturally found in intestinal mucosa of mice and it is widely used as an effective vehicle in the delivery of foreign antigen (6-8). We developed a TMEV based vaccine model that can generate immune response against a model antigen and weakly immunogenic tumor antigen rat-neu (9). Furthermore, we also observed that this TMEV based vaccine can be successfully used in treating mouse models of melanoma and breast cancer. In the same study we observed that this TMEV based vaccine delayed tumor outgrowth and increased survival of animals with implanted breast tumors (9). We modified this virus to deliver multiple immunogenic peptides C3, L3 and N1. Ability of this virus based vaccine targeting multiple tumor antigens to induce local immune responses when delivered through intramammary route was tested using virus itself as a vaccine model or in combination with peptide vaccine as a prime boost strategy. As shown in Fig. 11A and Fig. 12, we observed that Ima immunization of BALB/c mice with TMEV virus encoding peptides specific for multiple tumor antigens induced effective local immune responses against target antigens when the virus was delivered with prime boost strategy compared to delivery of virus alone as a vaccine. We also observed delivery of vaccine using prime boost strategy induced systemic immune responses. As shown in Fig. 11B, significant increase in IFN-γ production by splenic CD8 T cells was observed in mice that were immunized with TMEV vaccine using prime boost strategy. In the next step, we determined whether this prime boost strategy of vaccination will result in inhibition of spontaneous tumor growth in neu-transgenic mice. As shown in Fig. 13, we observed that this strategy of vaccination did not result in delay in the spontaneous tumor growth in neu-transgenic mice. Currently, we are testing the effectiveness of multiple booster doses of virus vaccine in combination with prime boost strategy to delay the spontaneous tumor growth in neu-transgenic mice. Also, different time points of intramammary immunizations instead of immunizing mice on week 10 and week

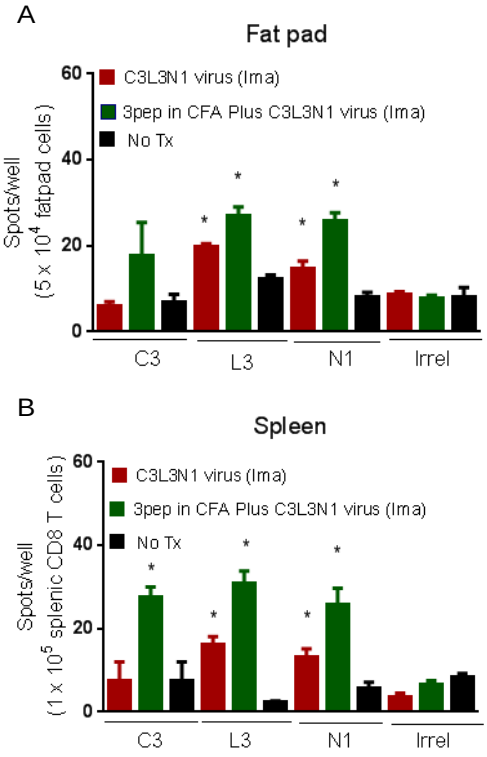

Figure 11. Intramammary immunization using prime boost strategy induces local antigen-specific immune responses in BALB/c mice. Mice were immunized with one dose of multi-peptide vaccine C3L3N1 in IFA (day 0) followed by immunization with Theilers virus encoding C3, L3 and N1 via intramammary route. Single cell suspensions obtained from fat pad cells (A), CD8 T cells obtained from spleen (B) were analyzed for peptide-specific responses using ELIspot. Shown are the results of IFN-γ production by mammary fat pad cells (A) and splenic CD8 T cells (B) in response to C3, L3 and N1 peptides pulsed P815 cells. Data is shown as mean (±s.e.m) of triplicates and is the representative of one of three experiments with similar results. *p<0.05 Vs. No Tx

12 is being tested to determine the effect of prime boost strategy in preventing the development of spontaneous tumors in neu-transgenic mice.

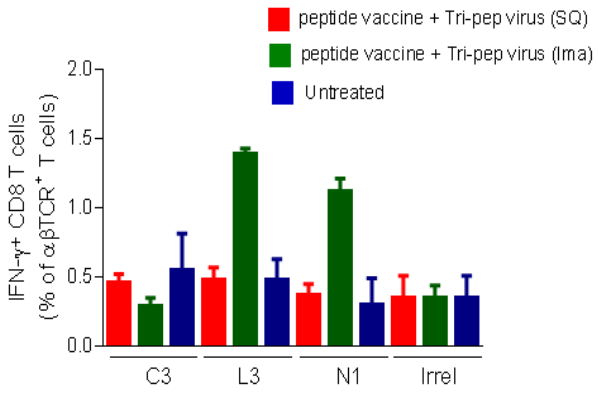

Figure 12. Intramammary immunization using prime-boost strategy induces local antigen-specific immune responses. BALB/c mice were immunized with a multi-peptide vaccine C3L3N1 via intramammary route and immunized with one dose of multi-peptide vaccine C3L3N1 in IFA (day 0) followed by immunization with Theilers virus encoding C3, L3 and N1 via intramammary route. Single cell suspensions obtained from mammary fat pads were stimulated in vitro peptide (C3 or L3 or N1) pulsed P815 cells 6 hrs. in the presence of Golgiplug. Cells were fixed using BD cytofix/cytoperm fixation/permeabilization kit and analyzed for lymphocyte markers and intracellular IFN-γ. Data is shown as mean (±s.e.m) of triplicates and is representative one of three independent experiments with similar results.

The Key Research Accomplishments

- Developed a picornavirus based vaccine model that can be used in cancer immunotherapy (9).
- Developed attenuated live virus vaccine targeting multiple tumor antigens that can effectively mobilize CD8+ cellular immunity to mammary fat pads up on intramammary immunization.
- Identified a unconventional T cell population (CD4+CD8+ T cells) that are localized in mammary fat pads and further analysis suggests that their function could be similar to cytotoxic T cells.
- Identified that immune cell subsets associated with normal breast mucosa are similar to normal gut mucosa.

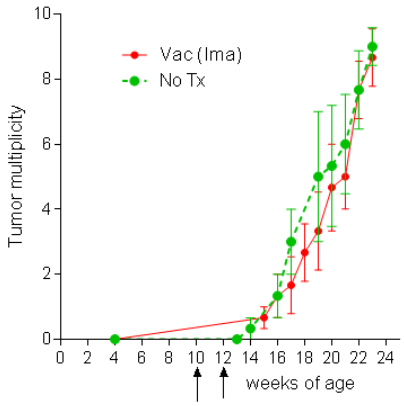

Figure 13. Intramammary immunization using prime-boost strategy did not delay spontaneous neoplasms in neu transgenic mice. BALB/c-neu transgenic were immunized with one dose of multi-peptide vaccine C3L3N1 in IFA (at week 10) followed by immunization with Theilers virus encoding C3, L3 and N1 (at week 12) via intramammary route as shown by arrows. All animals were monitored for tumor appearance by manual examination of the mammary glands every 5d. Measurable masses >2mm diameter were regarded as tumors. Data is shown as mean number of tumors (tumor multiplicity) in each group which is represented by points. These experiments were repeated twice with similar results.

Reportable outcomes:

None

Presentations at meetings:

None

Publications in scientific journals:

1. Pavelko KD, Bell MP, Karyampudi L, Hansen MJ, Allen KS, Knutson KL and Pease LR (2013). The epitope integration site for vaccine antigens determines virus control while maintaining efficacy in an engineered cancer vaccine. Mol Ther. 2013 May; 21(5):1087-95. doi: 10.1038/mt.2013.52. Epub 2013 Apr 9.

Conclusions

Our goal is to develop preventative breast cancer vaccine. Given the fact that breast cancer originates from mucosal tissue and it is well known that mucosal tissue associated with gut is endowed with intact immune system, we proposed, in this grant, to study the immune effectors associated with breast mucosa. Based on this information our goal was to develop strategies to educate breast mucosa using different vaccination approaches. The results obtained from studies done in the first year of this grant showed that a) majority of immune cells in breast mucosa are T cells and further analysis revealed that these are unconventional double positive T cells which have functions similar to cytotoxic T cells as evident from their cytokine profile, b) it is possible to generate immune responses in the breast mucosa using the peptide and prime boost vaccination strategies. In the second year of this grant we plan to a) understand the effect of different reproductive factors in altering the immune subsets of breast mucosa b) characterize and define the role of breast mucosa associated unconventional double positive T cells in protection against breast cancer, and c) identify optimal intramammary vaccination strategy that can prevent the breast cancer in transgenic mouse models of human breast cancer.

Principal Investigator Statement:

Overall, the progress of this project has been excellent to date. Our staff, collaborators, and I are excited and optimistic about the refinements that we have made to the project. The aims that we have chosen are novel, cutting edge, and challenging to us. In the initial part of the project the movement forward was challenging due to troubleshooting that had to be done to get the immune cell subsets from mammary fat pads of mice as this is completely unexplored area of research. Eventually these techniques were standardized. Also, in the beginning of this year (2013) our group started relocating from Mayo Clinic, Rochester, MN to Vaccine and Gene Therapy Institute of Florida, Port St. Lucie, FL. As a result of this, our efforts to complete some of the tasks proposed for year 1 in statement of work were slowed down, specifically the tasks that were related to the studying of the effect of different reproductive factors in altering immune subsets of breast mucosa, as we were relocating all our mouse colonies from Mayo Clinic to Vaccine and Gene Therapy Institute of Florida. However, this delay was covered by the theilers virus based vaccine studies that we had developed with our collaborators which lead to a publication and this virus based vaccine was modified and tested in the experiments proposed in this grant. Our group and our collaborators are excited about the use of this virus based vaccine model along with a peptide vaccine model we have developed. Upon completion of the studies that were proposed in the second year of this grant, we anticipate that we will be in a position to test a prime boost vaccine strategy as a preventative breast cancer vaccine in future studies.

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Appendices (one publication)

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The Epitope Integration Site for Vaccine Antigens Determines Virus Control While Maintaining Efficacy in an Engineered Cancer Vaccine

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Picornaviruses have been developed as potential therapies for gene delivery and vaccination. One drawback to their use is the potential for recombination and viral persistence. Therefore, the engineering strategies used must take into account the possibility for virus escape. We have developed Theiler's murine encephalomyelitis virus (TMEV) as a potential vaccine vector for use in immunotherapy. This study shows that insertion of a vaccine epitope at a unique site within the TMEV leader protein can dramatically increase the type I interferon (IFN) response to infection and promote rapid viral clearance. This live virus vaccine maintains its ability to drive antigen-specific $CD8^+$ T-cell responses to a model antigen as well as to the weakly immunogenic tumor antigen Her2/neu. Furthermore, the epitope integration site does not affect the efficacy of this vaccine as cancer immunotherapy for treating models of melanoma and breast cancer as demonstrated by delayed tumor outgrowth and increased survival in animals implanted with these tumors. These findings show that an attenuated virus retaining limited ability to replicate nonetheless can effectively mobilize $CD8^+$ cellular immunity and will be important for the design of picornavirus vectors used as immunotherapy in clinical settings.

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INTRODUCTION

Engineered picornaviruses have been developed as vaccine vectors for immunotherapy because of their inherent immunogenicity and relatively small genomes that can be easily manipulated using standard techniques. The harnessing of picornaviruses for immunotherapeutic purposes has been attempted using several viruses within this family including enterovirus,[1] coxsackievirus,[2] rhinovirus,[3] and cardiovirus.[4–6] However, the enthusiasm for using these viruses as vaccine vectors has been tempered by concerns regarding their inherent genomic instability and the prospect of reversion to wild-type sequences. Consequently, several studies have focused on engineering new picornavirus vectors that retain the engineered virus identity.[7,8]

We have recently identified the potential of the cardiovirus Theiler's murine encephalomyelitis virus (TMEV) as a vaccine candidate for immunotherapy.[6] We engineered vaccine antigens into the leader sequence of TMEV and demonstrated that a virus integrated epitope could drive strong $CD8^+$ T-cell responses and inhibit tumor outgrowth after treatment with engineered TMEV virus. The leader protein is unique among picornavirus proteins in that it is translated before the capsid proteins by virtue of its placement with the genome. The leader protein has been linked to immunosuppression through its ability to inhibit type I interferon (IFN) signaling which is necessary to evade innate immune effectors.[9,10] Mice with the appropriate major histocompatibility complex class I molecules subsequently clear the virus through adaptive immune mechanisms,[11,12] notably the $CD8^+$ T-cell response[13–15] and remarkably, the activation of this T-cell response occurs in the absence of costimulation, CD4 help, tumor necrosis factor, or IFNγ.[16]

Furthermore, studies using cell lines deficient in type I IFN responsiveness have shown that TMEV absent the leader protein can replicate *in vitro* but fail to efficiently replicate *in vivo*.[17] Our studies showed that insertion of the vaccine epitope within the leader protein at an XhoI site attenuated the virus to a degree, suggesting that insertion of vaccine epitopes within the leader may also serve as a mechanism for attenuating the virus.[6]

Although viral replication efficiency is often a concern in the development of attenuated live virus vaccines, little is known about how attenuation can influence the effective response to engineered virus vectors. In this study, we have engineered TMEV vaccines that use unique insertion sites within the leader protein and consequently have unique replication properties *in vivo*. We find that the insertion site can influence viral clearance by promoting RAG independent mechanisms of clearance without affecting vaccine responses. This strategy provides a safe vaccine vector that can be rapidly cleared before reversion to wild-type virus yet promotes effective $CD8^+$ T-cell immunity to vaccine antigens.

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RESULTS

Derivation of wild-type and attenuated vaccine strains of TMEV from recombinant vectors

Previously, we developed a live TMEV vaccine through transfection of RNA transcripts generated from a modified TMEV cDNA clone.[6] To bypass the need for generating RNA transcripts, we cloned the complete genome of the Daniel's strain of TMEV[18] into a eukaryotic expression vector (pci-DA). The pci-DA clone was directly transfected into BHK cells and the resulting transfection resulted in the release of viral progeny. Using site-directed mutagenesis, we generated modified TMEV vectors that expressed a fragment of chicken ovalbumen ($OVA_{244–260}$) that included the H-2K^b restricted CD8$^+$ T-cell epitope SIINFEKL. This fragment was cloned into the pci-DA vector at positions 1074 and 1227 of the TMEV genome. These modified viruses encode small insertions after the third amino acid of the TMEV leader protein (p3-OVA8) and at the XhoI restriction site further downstream (XhoI-OVA8). Neither construct disrupted the coding frame after the epitope insertion site (**Figure 1a**).

After transfection into cells, virus was recovered and quantified by plaque assay. The wild type, p3-OVA8, and XhoI-OVA8 demonstrated similar plaque size and infectivity compared with the wild-type virus (data not shown). To test the virulence of the engineered strains, we used an MTT assay to assess killing of BHK cells after infection with a titrating dose of each virus. After 24 hours of infection, the p3-OVA8 virus had a modest decrease in virulence compared with the other two viruses. However, the virulence of the wild-type virus was notably higher after 48 hours of infection compared with the p3-OVA8 and XhoI-OVA8 viruses, demonstrating attenuation of both modified strains (**Figure 1b**).

One unique aspect of viruses within the cardiovirus genus is the presence of a leader protein that modulates early cytokine release from infected cells as a mechanism of immune evasion. We infected B6 fibroblasts *in vitro* with modified viruses to determine whether the insertion sites we chose would modulate the cytokine response. We found that the p3-OVA8 virus elaborated more messenger RNA for IFNβ and IFNα after 24 hours of infection *in vitro*; in contrast, the wild-type pci-DA virus and XhoI-OVA8 virus elaborated an increased amount of IL-6 compared with the p3-OVA8 virus and uninfected control (**Figure 1c**). This suggests that regions within the leader can be disrupted by insertion of vaccine antigen with consequences toward host immune responses.

To verify the potential for using modified TMEV as therapy in humans, we infected human dermal fibroblasts and assessed their viability by MTT assay after 48 hours of infection. Similar to what we observed in mouse cells, we found that the p3-OVA8 and XhoI-OVA8 viruses were less virulent toward human cells when compared with the pci-DA wild-type virus (**Figure 1d**). Furthermore, we asked whether the innate immune response to infection of human cells could be modulated by using these viruses. We infected human fibroblasts for 24 hours and assessed the induction of human IFNβ and human IL-6 transcripts. We found that the p3-OVA8 and XhoI-OVA8 viruses elicited increased IFNβ in response to infection and that each of the three viruses responded with a unique IL-6 expression profile, supporting our hypothesis that the site of epitope integration can also modulate the innate immune response to viral infection in human cells.

Two distinct leader insertion sites modulate viral clearance

Clearance of TMEV from the CNS is determined by the H-2D region of major histocompatibility complex. B6 (H-2D^b) mice are resistant to persistent infection and clear virus, where FVB (H-2D^q)

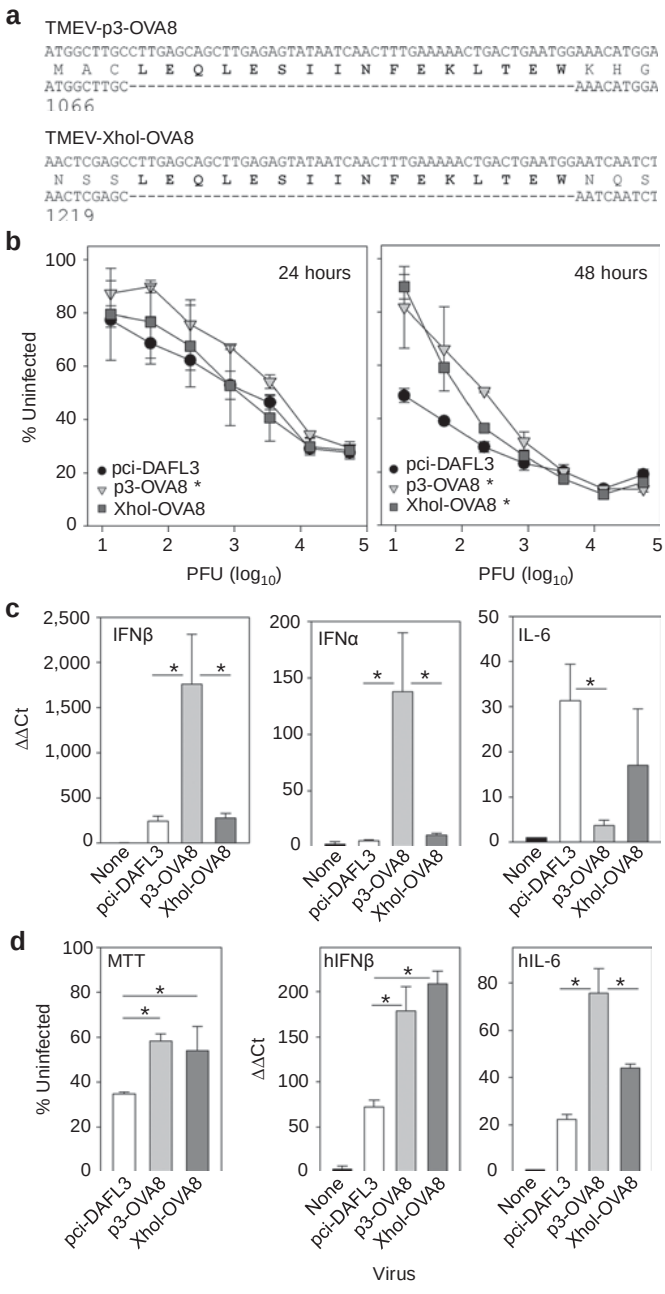

Figure 1 *In vitro* characterization of TMEV vaccines. (**a**) Amino-terminal sequence of TMEV L protein for the p3-OVA8 and XhoI-OVA8 viruses. $OVA_{253–268}$ was engineered into the wild-type TMEV expression vector by site-directed mutagenesis. (**b**) Killing of BHK cells by engineered TMEV viruses at 24 and 48 hours after infection. Triplicate samples were assessed for MTT metabolism. Data are presented as the percentage of uninfected cell signal ±SD (*P < 0.05, versus pci-DA by Holm-Sidak method). (**c**) IFNβ, IFNα, and IL-6 gene regulation in response to infection with modified TMEV viruses for 24 hours (*P < 0.05 by Holm-Sidak method). (**d**) MTT metabolism, human IFNβ (hIFNβ), and human IL-6 (hIL-6) gene regulation after infection of human dermal fibroblasts (*P < 0.05 by Holm-Sidak method). PFU, plaque-forming units.

mice fail to clear virus and therefore develop persistent infection. To determine how efficiently modified TMEV viruses can be cleared, we intracranially infected B6 mice for 3 and 6 days and FVB mice for 21 days to determine relative virus RNA levels. Three days after infection with pci-DA, p3-OVA8, or XhoI-OVA8 virus all mice had detectable levels of TMEV transcripts compared with uninfected mice. This level was reduced in all three groups at day 6, demonstrating that all three viruses replicated to some extent in the brain (**Figure 2a**). The p3-OVA8 virus never reached the RNA levels of the wild-type and XhoI-OVA8 viruses, demonstrating that this virus does not replicate as efficiently *in vivo*. Furthermore, infection of susceptible FVB mice reveals that the wild-type virus persists; however, the XhoI-OVA8 and p3-OVA8 RNA levels were reduced, with the p3-OVA8 being near the detection threshold (**Figure 2a**).

To determine the virus replication fidelity, we amplified cDNA derived from infected animals in a manner that allowed us to discriminate the engineered viruses by amplicon size. Virus recovered from 6 day wild-type–infected B6 mice generated a single band that was consistent with the predicted size of 326 base pairs. In two out of four animals infected with p3-OVA8, viral RNA was detected and amplicons were consistent with the insert size of 377 bp and sequencing revealed identity to the engineered virus sequence (**Figure 2b**). All XhoI-OVA8 infected animals had amplified products consistent with the modified virus as well as two less prominent bands. To determine whether this represented virus that had reverted to wild type, we sequenced the most prominent product and the smaller of the two amplified products marked in **Figure 2b**. The major product was identical to the cDNA clone that was used to generate the modified virus. Sequencing of the smaller band revealed a deletion of the coding sequence that truncates the final six amino acids of the ovalbumen insert (**Figure 1a**); however, wild-type revertants were not detected.

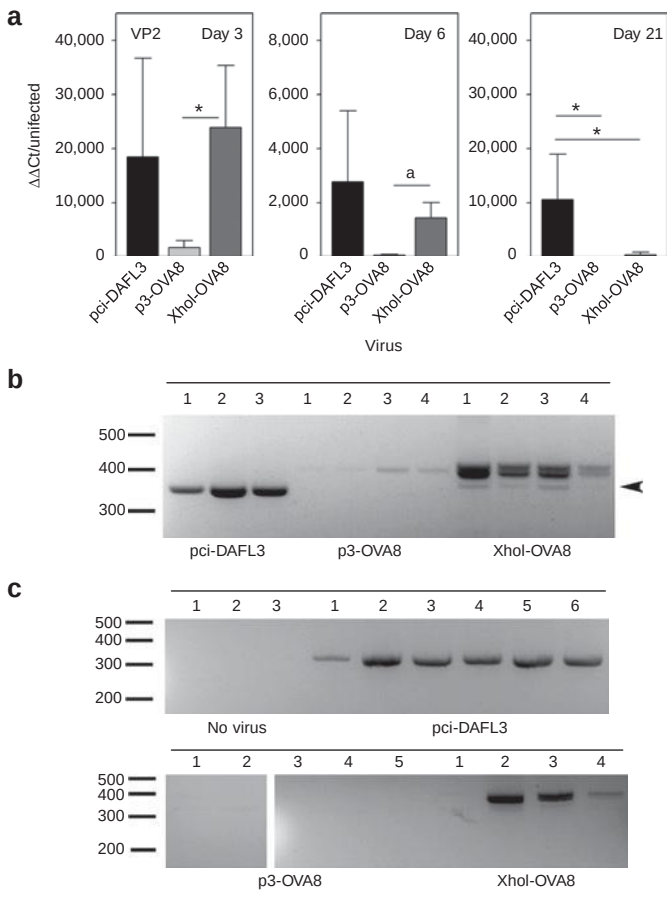

Figure 2 Infection with engineered TMEV. (**a**) Relative virus levels as determined by semiquantitative RT-PCR for TMEV viral protein 2 (VP2) in mice intracerebrally infected with TMEV viruses. Day 3 and 6 infected data represent infection of C57BL/6 mice and day 21 data represent infection of FVB mice (fold increase from uninfected CNS ±SD, $n = 5$ per group at day 3, $^{*}P < 0.05$ by Holm-Sidak method, $^{a}P < 0.05$ by *t*-test). (**b**) RT-PCR amplification across antigen insertion site of RNA recovered from C57BL/6 mice infected for 6 days from **a**. Arrow indicates deletion mutant. (**c**) Analysis of RT-PCR products derived from FVB mice infected with pci-DA, p3-OVA8, and XhoI-OVA8 for 21 days. All bands were isolated and sequenced to confirm identity of wild-type virus, modified TMEV virus or to identify deletion mutants. RT-PCR, reverse transcriptase PCR.

This strategy was repeated using the 21-day–infected RNA from FVB animals. Size and sequence of amplicons verified the recovery of wild-type virus in all six animals infected with pci-DA. No virus was detected in mice infected with the p3-OVA8 virus and three out of four XhoI-OVA8 animals had detectable levels of virus. In the FVB strain, wild-type and XhoI-OVA8 virus PCR fragments matched the sequence of the infecting virus. All FVB animals had detectable titers of anti-TMEV antibody, verifying that all animals were infected with the three viruses (data not shown).

Clearance of the p3-OVA8 vaccine strain is mediated through type I IFNs

The clearance of p3-OVA8 virus and the generation of a strong type I IFN response suggest that innate immunity may be sufficient for eliminating this virus. We intracerebrally infected RAG$^{-/-}$ mice with wild-type TMEV or our two OVA8-expressing vaccine strains to determine whether adaptive immunity plays a role in viral clearance. Although these mice lack T and B cells, the innate immune response in these animals is still intact, providing an opportunity to determine whether innate immunity is sufficient for clearing virus. After infection, mice were monitored for 28 days for signs of infection, weight loss, and fecundity. The wild-type and XhoI-OVA8 viruses developed a severe disease with noticeable weight loss by day 14 in both groups. All RAG$^{-/-}$ animals infected with pci-DA or XhoI-OVA8 were killed by day 16 or 19 when mice became moribund (**Figure 3a**). The p3-OVA8 infected mice did not exhibit weight loss or morbidity and survived through the 28 day monitoring point.

As the p3-OVA8 infected mice survived, we asked whether infectious virus might still be present in the CNS. We performed plaque assays on CNS homogenates derived from each of the RAG$^{-/-}$ animals infected with pci-DA, XhoI-OVA8, and p3-OVA8 viruses. We found high titers of pci-DA or XhoI-OVA8 virus in the RAG$^{-/-}$ CNS infected with each of these viruses. No detectable virus plaques were observed in monolayers exposed to CNS homogenates from p3-OVA8 infected RAG$^{-/-}$ mice, similar to cells exposed to an uninfected CNS homogenate (**Figure 3b**). Finally, RNA was isolated from the CNS to determine whether RNA levels were consistent with infectious titers. pci-DA and XhoI-OVA8 RNA levels were consistent with data obtained from plaque assays.

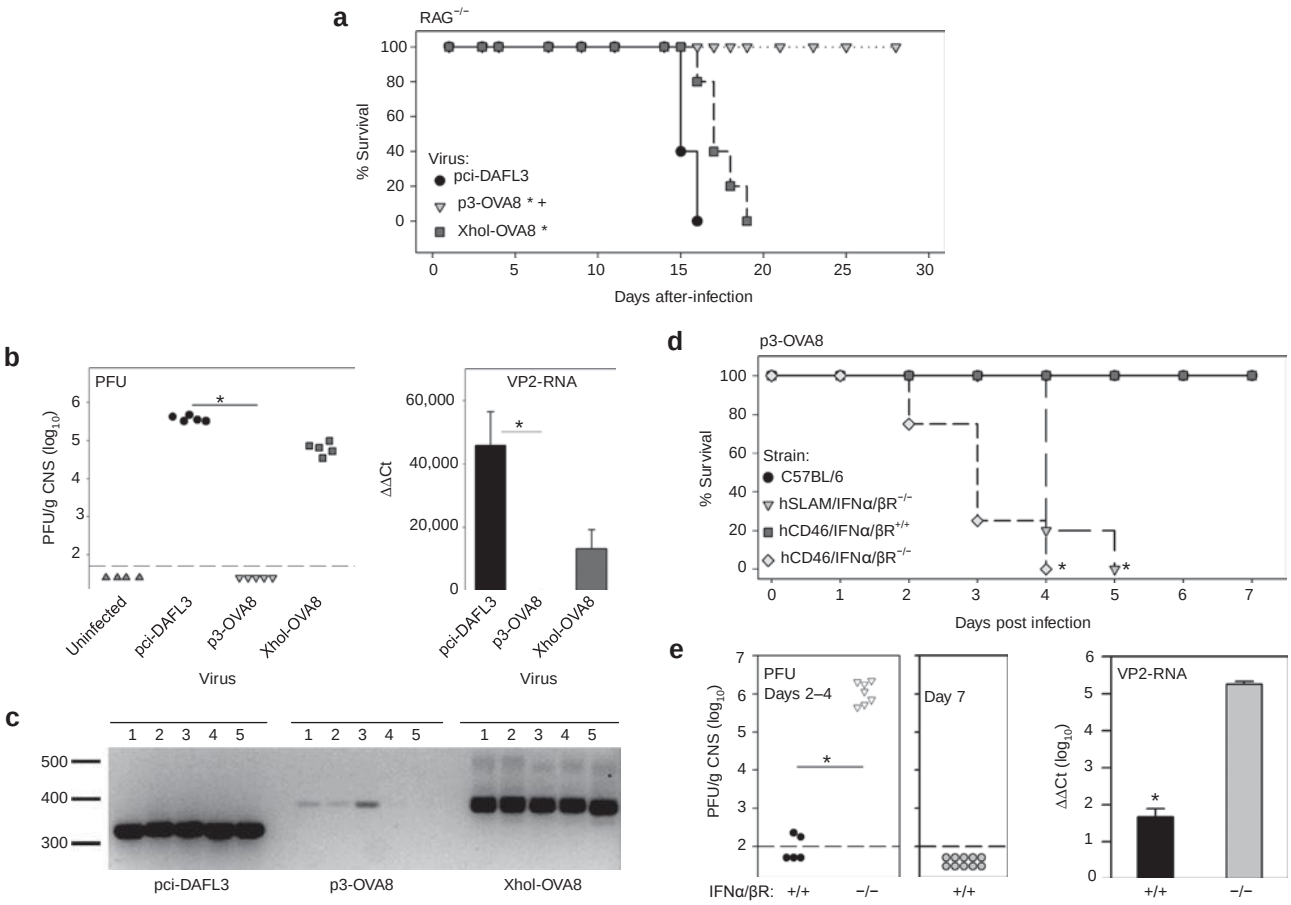

Figure 3 Adaptive immunity is not required for clearance of p3-OVA8. (**a**) Kaplan–Meier survival analysis in RAG$^{-/-}$ mice that were infected with pci-DA, p3-OVA8, and XhoI-OVA8 ($P < 0.05$ by Holm-Sidak method versus *pci-DA or $^{+}$XhoI-OVA8). (**b**) Virus titers and viral RNA levels from CNS homogenates recovered from RAG$^{-/-}$ mice analyzed in **a** (*$P < 0.05$ Dunn's method). (**c**) Amplification of RNA recovered from the CNS of animals in **a** to determine identity of infectious virus. All bands were verified by sequencing to confirm virus identity. (**d**) Kaplan–Meier survival analysis of IFNα/β receptor knockout mice infected with p3-OVA8 (*$P < 0.05$ by Holm-Sidak method versus C57BL/6 or hCD46/IFNα/βR$^{+/+}$). (**e**) Virus titers and viral RNA levels in CNS tissues recovered from animals in **d** (*$P < 0.05$ by *t*-test). PFU, plaque-forming units; VP2, viral protein 2.

Low levels of p3-OVA8 RNA were detected in homogenates from infected animals, demonstrating that low levels of virus were present (**Figure 3b**). To verify the fidelity of each of these viruses after infection, cDNA derived from the CNS was sequenced, demonstrating that all the infected animals retained viruses with their respective engineered viral genome (**Figure 3c**).

As the p3-OVA8 virus could be cleared in the absence of T or B cells, we asked whether clearance was mediated through the type I IFN pathway. We infected IFNα/β receptor knockout mice with p3-OVA8 virus and monitored for weigh loss and morbidity. As observed previously, both C57BL/6 and littermate control animals displayed no signs of overt disease. In contrast, two different strains of IFNα/βR$^{-/-}$ mice quickly lost weight and succumbed to viral infection within 5 days after infection (**Figure 3d**), demonstrating the importance of this pathway for clearance of p3-OVA8. To verify the presence of live virus, we performed plaque assays on recovered CNS homogenates and found extremely high titers of p3-OVA8 virus in the IFNα/βR$^{-/-}$ animals that were infected for 2–4 days, in comparison with littermate controls or C57BL/6 animals infected for 2–4 or 7 days (**Figure 3e**). We verified the presence of viral RNA in all animals by semiquantitative reverse transcriptase PCR (**Figure 3e**).

p3-OVA8 and XhoI-OVA8 drive CD8 T-cell immunity that delays melanoma outgrowth

By engineering major histocompatibility complex class I epitopes into the leader sequence of TMEV, we have identified a strategy that mediates virus control and provides an added level of safety for our live virus vaccine. However, it is unclear whether attenuation of the virus in this manner will be detrimental to its use as a T-cell vaccine. To determine whether the p3-OVA8 and XhoI-OVA8 viruses can effectively drive proliferation of antigen-specific T cells, we determined how effectively these viruses drive proliferation of transgenic CD8^{+} T cells specific for OVA8. We found that infection with either the p3-OVA8 or the XhoI-OVA8 viruses drives proliferation of the T-cell population (**Figure 4a**). Furthermore, both viruses drove a significant dilution of Carboxyfluorescein diacetate succinimidyl ester (CFSE) indicating that these viruses were able to promote proliferation of T cells in response to infection with p3-OVA8 or XhoI-OVA8 when compared with pci-DA infection (**Figure 4a**).

By demonstrating that antigen-specific T cells respond to our vaccines, we establish that these vectors can sufficiently present vaccine antigens and promote proliferation of a transgenic T-cell population. However, in the absence of a high-frequency T-cell

receptor, we wanted to determine whether these vaccines drive de novo CD8 T-cell responses in naïve animals. To test this, we intracerebrally infected mice with pci-DA, p3-OVA8, and XhoI-OVA8 viruses and analyzed the CNS infiltrating lymphocytes after 6 days of infection. We compared the vaccine antigen-specific $CD8^+$ T-cell responses to the endogenous $VP2_{121–130}$ response typically observed in H-2D^b mice. All viruses elicited a strong endogenous viral response after infection and no response was detected using the control tetramer $E7_{49}$ (**Figure 4b**). The p3-OVA8 and XhoI-OVA8 elicited OVA8 responses compared with the pci-DA wild-type virus, demonstrating their ability to drive a strong polyclonal response to this model antigen.

As these vaccines drive responses to the model antigen, we evaluated whether these vaccines would inhibit tumor outgrowth when used as immunotherapy. We established melanoma tumors in mice and allowed them to grow in the absence of intervention for 9 days. We treated tumor-bearing mice with p3-OVA8, XhoI-OVA8 or wild-type pci-DA virus and monitored changes in tumor size. Both viruses inhibited the outgrowth of the B16-OVA tumors and delayed outgrowth by day 7 after treatment with the virus vaccine compared with pci-DA virus (**Figure 4c**). Furthermore, these viruses significantly increased survival by delaying the growth of tumors through the 25 day monitoring window (**Figure 4d**).

Her2/neu vaccines drive antigen-specific CD8 T-cell responses and inhibit breast cancer growth

Although we demonstrate the ability to drive directed CD8 T-cell responses to the model antigen ovalbumen and show that this response inhibits tumor outgrowth, the potential of this vaccine approach may only be realized if responses to weakly immunogenic tumor antigens can be attained. To address this, we generated TMEV vaccines that encoded an H-2K^d peptide derived from Her2/neu, p66.[19] We intracranially infected B6/Balb F1 mice with XhoI-p66 virus as well as the wild-type pci-DA. This allowed us to monitor the response to the H-2D^b $VP2_{121–130}$ peptide as well as the H-2K^d/p66 response by tetramer. CNS infiltrating lymphocytes were harvested after 6 days of infection. As expected, the wild-type TMEV infection yielded no response to H-2K^d/p66 or to the H-2K^d/RSV control peptide, but did respond to the endogenous VP2 antigen (**Figure 5a**). Infection with the XhoI-p66 virus yielded a weak, but significant CD8 response to the p66 peptide as measured by tetramer and no response to RSV demonstrating antigen specificity. Furthermore, the response to the endogenous $VP2_{121–130}$ peptide was present, similar to wild-type virus (**Figure 5a**).

Although the response to p66 could be detected in the F1 hybrids using the XhoI-p66 virus, we wanted to determine whether a p3-p66 virus and the XhoI-p66 viruses could induce antigen-specific $CD8^+$ T-cell responses in Balb/c mice by assessing their induction of IFNγ by ELISPOT. To address this, we infected Balb/c mice

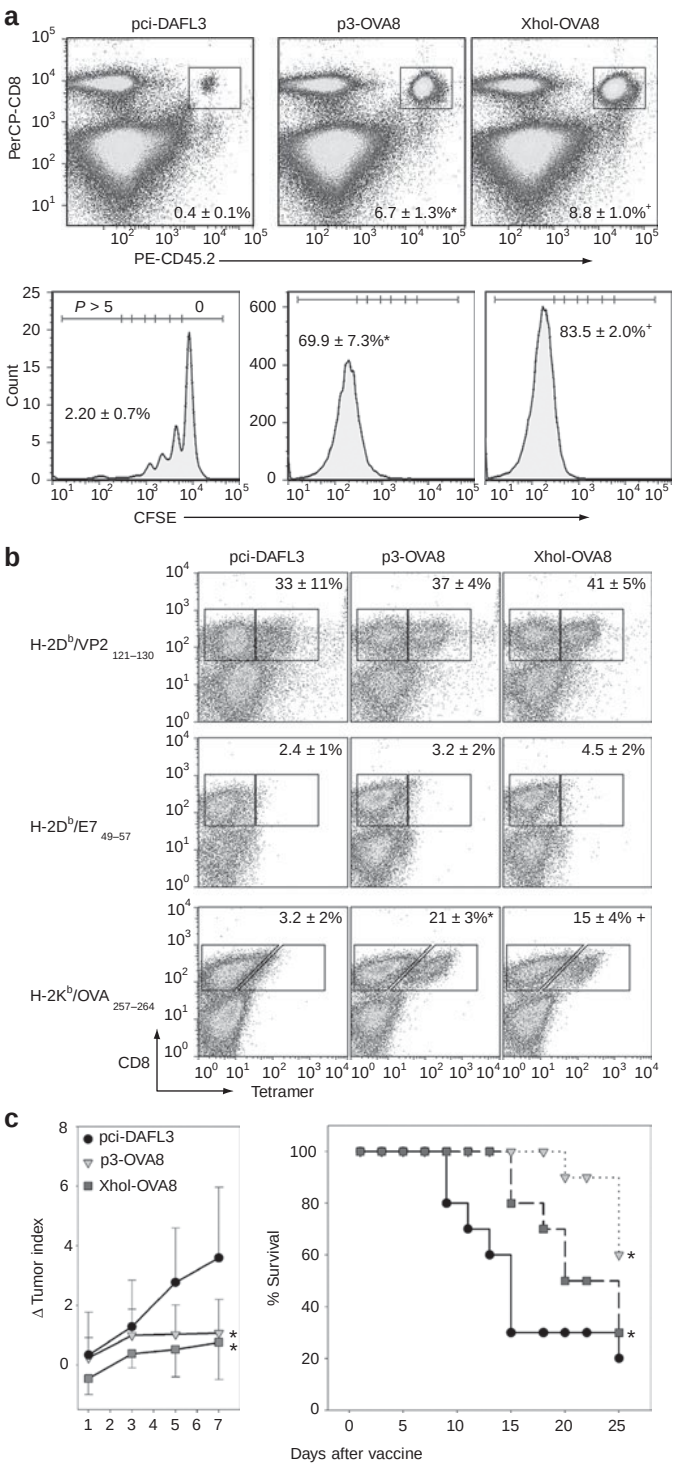

Figure 4 TMEV vaccines drive antigen-specific $CD8^+$ T-cell responses and reveal their potential as immunotherapy. (**a**) Recovery and proliferation of $CD45.2^+$ /$CD8^+$ OT-1 T cells from splenocytes of vaccine infected mice (n = 5 for all groups, % recovered cells ± SD). Cells were binned according to rounds of proliferation as determined by carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution. Data below are the percentage of cells that have gone through greater than five rounds of proliferation. *P < 0.05 pci-DA or XhoI-OVA8; ^+P < 0.05 versus pci-DA or p3-OVA8 by Holm-Sidak method. (**b**) Virus- and vaccine-specific $CD8^+$ T-cell responses determined by MHC class I tetramers in naïve C57BL/6 mice infected with TMEV vaccines for 6 days (percentage of $CD8^+$ T cells specific for VP2, E7 control or OVA ± SD). *P < 0.05 pci-DA or XhoI-OVA8; ^+P < 0.05 versus pci-DA or p3-OVA8 by Holm-Sidak method. (**c**) Delayed growth of established tumors and increased survival in mice challenged with the melanoma model tumor B16-OVA. Both p3-OVA8 and XhoI-OVA8 vaccines significantly delayed outgrowth as melanoma therapy by day 7 after treatment (*P < 0.05 versus pci-DA by Holm-Sidak) and increased survival during the 25 day observation period (*P < 0.05 versus pci-DA by Kaplan–Meier survival analysis with pairwise comparisons performed using Holm-Sidak method). MHC, major histocompatibility complex.

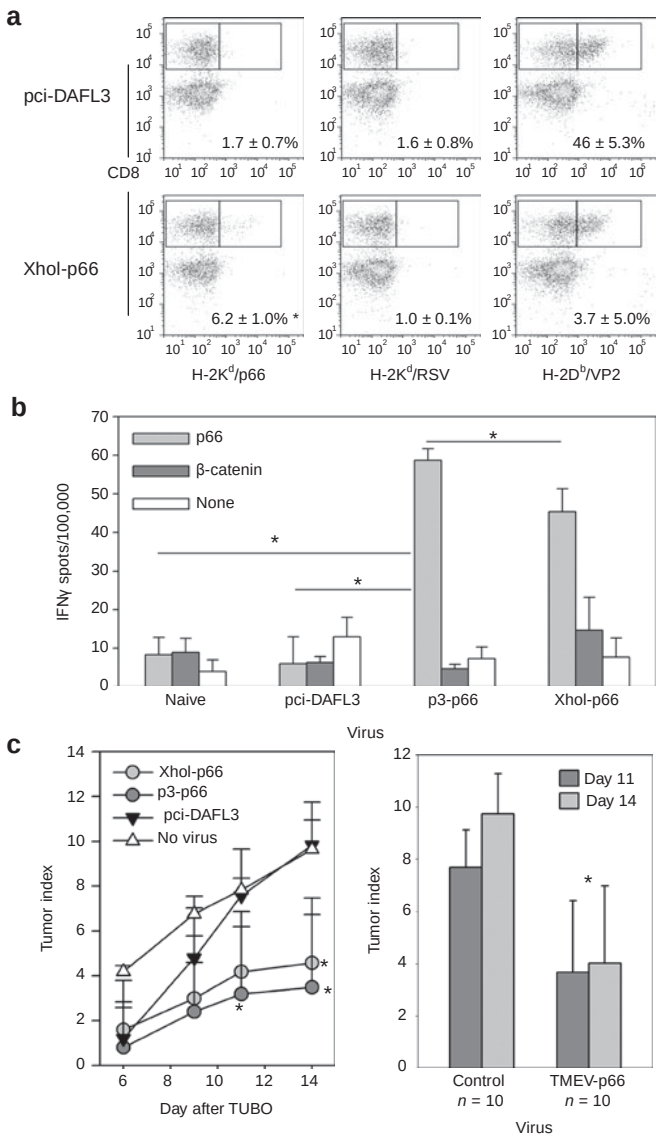

Figure 5 TMEV-p66 vaccines promote Her2/neu immunity and inhibit outgrowth of a breast cancer tumor line. (**a**) Her2/neu-specific CD8$^+$ T-cell responses in B6/Balb/c F1 mice (% of CD8$^+$ T cells specific for the Her2/neu peptide p66, RSV, or VP2 ±SD, n = 3 per group, *P < 0.05 by Holm-Sidak method). (**b**) ELISPOT assay for p66-specific IFNγ response in Balb/c mice splenocytes after immunization with TMEV-p66 vaccines, control virus or no virus (*P < 0.05 by Holm-Sidak method). (**c**) Delayed outgrowth of TUBO tumors in p3-p66 and XhoI-p66 vaccinated mice (right) at 11 and 14 days after tumor in p3-OVA8 vaccinated animals and at day 14 in XhoI-OVA8 vaccinated (*P < 0.05 by Holm-Sidak method). Nonregressors were combined as control group compared with the p3-p66– and XhoI-p66–treated TMEV-p66 group at 11 and 14 days (*P < 0.05 by Holm-Sidak method).

intraperitoneally with pci-DA, p3-p66, and XhoI-p66 viruses and assessed IFNγ responses 7 days after, using purified CD8$^+$ splenocytes. We find that both the p3-p66 and XhoI-p66 elicited a significant response to the relatively weak Her2/neu peptide, where naïve CD8$^+$ splenocytes and wild-type infected failed to respond (**Figure 5b**), demonstrating that both vaccines are able to elicit a targeted and specific CD8$^+$ T-cell response to this weak antigen.

As we demonstrate that this vaccine can elicit Her2/neu-specific CD8$^+$ T-cell immunity, we assessed its potential as a breast cancer vaccine by treating mice with our TMEV-p66 viruses before the transfer of Her2/neu$^+$ tumor cells onto the flank of Balb/c mice. After placement of the tumor, we monitored growth in the treated mice for 14 days after engraftment. We found that the p3-p66 treatment demonstrated a significant delay in outgrowth at day 11 and 14, where the XhoI-p66 significantly delayed outgrowth at day 14 (**Figure 5c**). The p3-p66 and XhoI-p66 demonstrated durable responses to the tumor with 3 out of 10 mice completely eradicating tumor, whereas all tumors grew in the control pci-DA and no treatment groups. As combined control or p66 vaccine groups, we find that there was a significant difference at both day 11 and day 14, with a decrease in tumor index in the p66 virus–treated groups (**Figure 5c**).

DISCUSSION

Our objective is to engineer a live virus vaccine for use as immunotherapy. Previous studies demonstrate the potential use of TMEV as a vector that induces CD8$^+$ T-cell responses against a model antigen and can suppress tumor growth *in vivo*.[6] In this study, we identify strategies for manipulating the TMEV genome in a manner that can have consequences on the cytokine response elaborated after live virus infection, the development of protective CD8$^+$ T-cell responses, and on live virus vaccine persistence. In addition, we demonstrate for the first time that TMEV can be used as a vector for driving CD8$^+$ T-cell responses toward a weakly antigenic tumor antigen after a single treatment without additional immune modulation. These findings will be important for the further development of this virus as a vector for immunotherapy.

In an effort to identify insertion sites into the TMEV genome that might render the vaccine more immunogenic, we used the XhoI restriction site of the TMEV leader protein as well as a unique site after the third amino acid of the leader. As the TMEV genome is translated as one large polyprotein that is subsequently cleaved, we preferably chose sites at the amino terminus to promote more efficient major histocompatibility complex class I presentation of the virus encoded antigens. Both of these virus vaccines drive proliferation and activation of CD8$^+$ T cells in a manner that demonstrates their potential for immunotherapy. However, we also identified some potential advantages for using the p3 insertion site when developing TMEV as a vaccine. One advantage of using the p3 vaccine is that infectious virus can be cleared in animals with severe immunodeficiency using protective mechanisms mediated through IFNα/β receptor signaling. However, attenuation of virulence does not inhibit the ability to drive CD8$^+$ T-cell responses using this approach. Although the increased IFNβ response may be directly responsible for viral clearance after infection, we speculate that modification of the cytokine milieu may also directly or indirectly affect the quality of the CD8$^+$ T-cell response. Studies have shown that type I IFN responsiveness by CD8$^+$ T cells can affect the quality of their effector function, responsiveness to recall antigens and antitumor activity.[20,21] Further studies will address how increases in the secretion of IFNβ in response to infection can alter the CD8 T-cell response.

Although we were able to identify two insertion sites that were amenable to the production of attenuated live virus, each virus elaborated a unique cytokine response after infection.

This suggests that a critical immune modulating function of the virus may be disrupted by insertion of $CD8^+$ T-cell epitopes into the leader protein and suggests a potential mechanism for differential cytokine production. Previously, it had been shown that the leader sequence of TMEV was important for inhibiting immediate/early IFNα/β production after infection.[9] This line of investigation went on to further determine that this activity was associated with a zinc finger motif at the amino terminus of the protein.[10] Our modification sites are immediately after the cysteine at amino acid position 3, an amino acid that is necessary for the formation of the zinc binding motif[22] and after amino acid position 54 of the TMEV genome. Although our virus constructs do not remove the necessary amino acid residues needed for binding zinc, the introduction of new sequences may interfere with its function as a modifier of IFNβ production, thus inhibiting the immune modulating capability of the leader protein. Of interest was the ability of the XhoI-OVA8 virus to induce expression of IFNβ after infection of human cells, suggesting that the ability of the virus to modulate human innate immunity may be unique compared with infection in mice. Consequently, future studies with aims toward clinical development may be more predictive if innate immune responses are assessed in human cell lines.

In addition to modulation of IFNα/β production after virus infection, we found that by engineering vaccine epitopes into the leader protein of TMEV, we could modulate IL-6 mRNA production after infection of primary fibroblasts. Previous studies have demonstrated that TLR3 responsiveness is necessary for driving proinflammatory cytokine responses after TMEV infection.[23] This was primarily mediated through interaction with viral RNA and subsequent activation of NFκB, a transcription factor necessary for the production of IL-6 and other proinflammatory cytokines. Although all of our viruses elicited a detectable amount of IL-6 after infection, the induction of this cytokine by the p3-OVA8 virus varied by species, with an increase in IL-6 induction observed in human fibroblasts compared with mouse. Whether this is related to how the leader protein functions in human cells is not clear at this time. Previous studies using TMEV have identified an interdependence between NFκB activation and viral replication as well as proinflammatory induction,[23–25] which would explain the IL-6 response to the wild-type virus infection. Although the precise mechanism for the lack of IL-6 production after infection with the p3-OVA8 virus in mouse cells is not known, one possibility is that increased type I IFN production inhibits viral replication and spread thereby decreasing the overall potential for activating NFκB and downstream proinflammatory cytokines. Although the precise mechanisms for modulation of cytokine responses to the engineered TMEV vaccines is not known, it is clear that these modifications can be used to modulate the immune response to infection in both human and mouse cells.

We found that the insertion of epitopes into the XhoI restriction site of TMEV renders infectious particles that are capable of driving antigen-specific T-cell responses; however, infection of C57BL/6 mice with the XhoI-OVA8 virus resulted in partial deletion of the vaccine epitope. Although this was a less prominent fraction of the amplified TMEV, it reveals the potential for virus instability and emergence of deletion mutants that may have a growth advantage *in vivo*. Several mechanisms have been identified as contributors to genomic variability in picornaviruses.[26] However, the ability to delete short genomic segments seems to be predominantly through the use of short direct repeats that act as parting and anchoring sites and allow the deletion of intervening genomic segments.[27] Although it is not known precisely how the deletion of TMEV genomic RNA occurs in our studies, the mechanism of deletion proposed by Pilepenko and colleagues is likely. Consequently, genomic virus constructs could be rationally designed to minimize these repeats thereby inhibiting the development of deletion mutants and enhancing stability and safety for use as immunotherapy.[28]

Several picornaviruses have been identified as potential treatments for human disease.[1,6,29,30] These have reached clinical trials as tumor oncolytics for the treatment of melanoma, breast cancer, and prostate cancer (http://clinicaltrials.gov/show/NCT00636558) as well as glioma and small cell lung cancer.[31,32] However, viruses within the cardiovirus genus have only recently been realized as potential therapeutics.[6,30] Controversy surrounding whether cardioviruses infect humans and exist naturally in humans has recently been addressed with the identification of a broad group of TMEV-like viruses that do not clearly produce clinical illness.[33] Although our findings use the murine cardiovirus TMEV, the sequence homology between TMEV and these human cardioviruses is extensive, suggesting that the principles identified here may be applicable to the development of human cardioviruses as therapeutics.

Seneca Valley virus has been developed and used as an oncolytic virus therapy for the treatment of small cell lung cancer and retinoblastoma.[30,32] Although its origin is not known, it is believed to have originated in swine, suggesting that species barriers for infectivity and use as therapeutics for human disease may not be critical within this genus of picornavirus.[34] TMEV was originally identified as a pathogenic virus in mice[35] and no cases of clinical disease have been attributed to TMEV infection in humans despite being extensively investigated in open laboratories since its discovery over 70 years ago. However, this does not necessarily preclude it for use as an attenuated live vaccine because TMEV has been shown to infect several human cell lines in culture.[23,24,36] Here, we have extended this finding to include human primary fibroblasts, further validating the potential for its use as a live attenuated vaccine. Finally, engineered TMEV may provide advantages over other human picornaviruses in that pre-existing antibodies that promote antibody-dependent enhancement of infection or that quickly neutralize virus may not be present to this murine virus.[37] These findings may be valuable for the development of new variants of this virus that could be used in future immunotherapies.

The data in this report demonstrate the potential for using TMEV as immunotherapy that can be used in a safe and directed manner. This attenuated virus retains a limited ability to replicate *in vivo* and provides a therapy that can drive strong $CD8^+$ T-cell immunity. Furthermore, this live virus vector promotes antitumor immunity that may be more potent than currently available therapies. The use of this safe and effective live virus vaccine lends promise to the future use of this and other cardioviruses as immunotherapy in clinical settings.

MATERIALS AND METHODS

Mice, cell lines, and reagents. C57BL/6, BALB/cByJ, CB6F1/J, FVB/NJ, B6.129S7-*Rag1*^{tm1Mom}/J, and C57BL/6-Tg(TcrαTcrβ)1100Mjb/J (OT-1) mice were purchased from Jackson Laboratory (Bar Harbor, ME). B6.SJL (CD45.1 congenic) mice were purchased from the National Cancer Institute mouse repository (Frederick, MD). Human CD45 and human SLAM transgenic mice deficient for the mouse IFNα/β receptor[38,39] were kindly provided by Dr Roberto Cattaneo (Mayo Clinic, Rochester, MN). Mice were infected intracerebrally (2×10^4 plaque-forming units) or intraperitoneally (2×10^5 plaque-forming units) with wild-type or modified TMEV viruses. All animals were housed in the Mayo Clinic Department of Comparative Medicine and cared for according to institutional and NIH guidelines for animals use and care.

BHK, L929, P815, B16-OVA, and TUBO cell lines were maintained in DMEM (GIBCO Invitrogen, Grand Island, NY) containing 10% fetal bovine serum (GIBCO Invitrogen). Media for B16-OVA was supplemented with 10 mg/ml G418 (Life Technologies, Carlsbad, CA) to maintain expression of the ovalbumen transgene. Fibroblasts from C57BL/6 mice were generated as described previously.[40] Human dermal fibroblasts were obtained from Lonza (Basel, Switzerland) and were maintained in RPMI (GIBCO) supplemented with 10% fetal bovine serum. MTT assays for cell death were performed on cells as described.[41]

PerCP-labeled anti-CD45 and allophycocyanin-labeled antimouse CD8 were purchased from BD Biosciences (San Diego, CA). PE-labeled tetramers for H-2K^d/p66, H-2K^d/RSV, H-2K^b/OVA$_{257}$, and H-2D^b/E7$_{49}$ were either purchased directly or custom manufactured by Beckman Coulter (Brea, CA). PE-labeled H-2D^b/VP2$_{121}$ tetramers were kindly provided by the NIH Tetramer Core at Emory University (Atlanta, GA).

Generation of TMEV expression plasmids and infectious virus. To generate the eurkaryotic expression vector for generating modified TMEV, we recloned the ClaI/XbaI fragment from the DAFL3 genomic clone for the Daniel's strain of TMEV[18] into the eukaryotic expression vector pci-Neo (Promega, Madison, WI). Before cloning, we introduced a new ClaI site into the multiple cloning site of pci-Neo to facilitate direct ligation into the vector. This vector has a CMV promoter sequence that allows robust expression of insert fragments after transfection into eukaryotic cells. Modified TMEV vectors with epitope insertions were generated by using site-directed mutagenesis (QuikChange II Site-Directed Mutagenesis Kit; Agilent, Santa Clara, CA) to introduce the nucleotides 822–869 (Accession no. V00383.1) encoding amino acids 253 through 268 from chicken ovalbumin (AAB59956) and nucleotides 292–336 (NM_017003.2) encoding amino acids 63 through 77 from the rat Her2/neu oncogene (NP058699). All custom made oligonucleotides were manufactured by Integrated DNA Technologies (Carolville, IA). All virus encoding plasmids were verified by sequencing at the Mayo Clinic Advanced Genomics Technology Center.

To generate infectious virus, we transfected 30 µg of vector DNA that contained the wild-type or modified virus sequences into 10^7 BHK cells using electroporation. Media was changed on day 1 and 4 after transfection and cells were monitored daily for signs of cytopathic effect. By day 7, the generation of productive virus infection was noted by the presence of mostly rounded cells floating in media with few cells remaining adherent. Cells and supernatant were collected, sonicated, and spun to yield a clarified supernatant. Supernatants were titered by plaque assay before use in vaccine experiments. Viruses were tested for stability by serial passage on BHK monolayers. After seven passages on BHK monolayers, the p3-OVA8 and XhoI-OVA8 viruses maintained the modified insert and no wild-type virus genome was detected by reverse transcriptase PCR across the epitope insertion sites.

Quantitation of cytokines and virus. To determine the levels of viral RNA, we used real-time reverse transcriptase PCR as described previously.[6] Briefly, RNA was isolated from cells or CNS homogenates using Trizol (Invitrogen, Carlsbad, CA), cDNA was generated using the Superscript III cDNA synthesis kit (Invitrogen) and reaction was set up according to the Fast SyBR Green Master Mix Kit (Applied Biosystems, Foster City, CA). We used the ΔΔCt method to calculate fold change over uninfected samples using actin as a reference.[42] We used the following primer sets to analyze virus and cytokines: mouse and human actin (F-5′CTGGCACCACACCTTCTACAATG AGCTG and R-5′GCACAGCTTCTCTTTGATGTCACGCACGATTTC), viral protein 2 (VP2) of TMEV (F-5′TGGTCGACTCTGTGGTTACG and R-5′GCCGGTCTTGCAAAGATAGT), mouse IL-6 (F-5′CAAAGCC AGAGTCCTTCAGAGAGATACA and R-5′ AGGAGAGCATTGGAAA TTGGGGTAGGAA), mouse IFNβ (F-5′ TGGGTGGAATGAGACTATT GTTGTAC and R-5′TGAGGACATCTCCCACGTCAATCTTT), and mouse IFNα (F-5′ CCCTCCTAGACTCATTCTGCA and R-5′CTCTGA CCACCTCCCAGGCAC). The primers for IFNα amplified a conserved region that is shared between IFNα 1, 2, 5, 6, 7, 9, 11, 12, and 13. The primers used for amplification of human genes were: human IL-6 (F-5′GGTACATCCTCGACGGCATCT and R-5′GTGCCTCTTTG CTGCTTTCACAC) and human IFNβ (F-5′AGGATTCTGCATTACCTG AAGG and R-5′GGCTAGGAGATCTTCAGTTTCG).

To verify modified TMEV insert fidelity by reverse transcriptase PCR, we amplified sequences within the leader sequence between positions 963 and 1288 of the cloned DA strain of TMEV[18] using the following primers: forward (5′GAGACACGTCGCAAGTGCTTTACACCTG) and reverse (5′GTTCCATGACAATATCGCGTACGAGCG). These primers amplified a 326 base pair fragment using the wild-type pci-DA virus and included the cloned insert sequence in modified TMEV strains. Amplified fragments were gel purified and sequenced to verify sequence identity.

Brains and spinal cord from mice infected with pci-DA, p3-OVA8, or XhoI-OVA8 were homogenized, sonicated, and clarified and tested for viral titer using plaque assays described previously.[43]

Lymphocyte analysis. CFSE staining and analysis of OT-1 cells was performed as described previously.[15] Briefly, OT-1 CD8$^+$ T cells were purified by magnetic column isolation using the mouse CD8a$^+$ T-cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Congenic CD45.1 mice received 1×10^7 purified CD8$^+$ T cells labeled with CFSE by intravenous transfer and were intraperitoneally infected with wild-type, p3-OVA8, or XhoI-OVA8 virus. After 48 hours, splenocytes were isolated and analyzed by flow cytometry for CD45.2, CD8, and CFSE content using FloJo Software (Tree Star, Ashland, OR).

To assess IFNγ responsiveness, CD8$^+$ T cells were isolated from Balb/c splenocytes 14 days after infection with TMEV viruses. Cells were assessed by ELISPOT as described previously.[19] The p66 peptide (TYVPANASL) and β-catenin peptide (SYLDSGIHS) were manufactured by Elim Biopharm (Hayward, CA).

Tumor experiments. B16-OVA tumor experiments were performed as described previously.[6] To assess protection from TUBO outgrowth, we transferred 2×10^5 cells into the right flank of Balb/c mice that were given TMEV vaccines 7 days before cell transfer. We monitored tumor growth by measuring tumor in two dimensions. Tumor index was calculated by the following formula: $(W \times H)^{1/2}$.

Statistics. All data were analyzed by one-way or two-way analysis of variance. Multiple comparisons were performed using the Holm-Sidak method for normally distributed data and by Dunn's method for nonparametric data. Data with two groups were compared by Student's *t*-test. The Kaplan–Meier survival analysis was performed by the Gehan-Breslow method with pairwise comparisons analyzed by the Holm-Sidak method. Significance was determined by $P < 0.05$. All statistical analysis was performed using SigmaStat3.1 software (Systat Software, San Jose, CA).

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